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Genetic variation in anti-herbivore chemical defences in an invasive plant

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Summary

1. Plants produce a variety of secondary metabolites such as flavonoids or tannins that vary in effectiveness against different herbivores. Because invasive plants experience different herbivore interactions in their introduced versus native ranges, they may vary in defence chemical profiles.

2. We subjected tallow tree (*Triadica sebifera*) seedlings from native (China) and introduced (US) populations to induction by leaf clipping or one of three Chinese caterpillars (two generalists and one specialist). We measured the concentrations of five flavonoids and four tannins in leaves produced before or after damage. We measured growth of caterpillars fed these leaves from plants of each induction treatment or undamaged controls.

3. Plants from introduced populations had higher flavonoids and lower tannins than plants from native populations, especially in new leaves following induction. Caterpillar responses to changing chemical concentrations varied in direction and strength, so overall performance varied from significantly lower (generalist *Grammodes geometrica*), unchanged (generalist *Cnidocampa flavescens*), to significantly higher (specialist *Gadirtha inexacta*) on introduced populations.

4. *Synthesis*. Together, such a trade-off in secondary metabolism in invasive plants and the effect on herbivores suggest divergent selection may favour different chemical defences in the introduced range where co-evolved natural enemies, especially specialists, are absent.

Key-words: constitutive and inducible defences, EICA, evolution of increased competitive ability, flavonoids, invasion ecology, tannins

Introduction

Invasive plants often escape suppression by co-evolved insect natural enemies from their native range (Enemy release hypothesis; Elton 1958; Maron & Vilà 2001) and may encounter novel herbivores in the introduced range (Strauss, Lau & Carroll 2006); therefore, their chemical defence against herbivory may differ between introduced and native ranges. For example, Joshi & Vrieling (2005) reported a higher concentration of pyrrolizidine alkaloids in introduced populations than that in native populations of *Senecio jacobaea*, which explained their lower resistance to specialist herbivores and higher resistance to generalist herbivores compared to native populations. Ridenour *et al.* (2008) found a greater resistance to herbivory in introduced populations of *Centaurea maculosa* than in native populations, partly due to a higher leaf concentrations of a biochemical defence compound precursor.

Trade-offs between plant defence and growth, types of chemical and mechanical defences, and constitutive and induced defences have been reported (see reviews by Stamp 2003 and Koricheva, Nykanen & Gianoli 2004). Given that invasive plants often have less damage by herbivores in their introduced ranges, they may reallocate resources from defence against natural enemies to growth and reproduction (the evolution of increased competitive ability hypothesis, or EICA hypothesis; Blossey & Nötzold 1995). Such trade-offs between defence and growth may explain why plants are more vigorous in their invasive range than in their native range. However, the overall results are mixed for tests of EICA and no clear pattern exists in the literature (see review by Bossdorf *et al.* 2005 and recent studies such as Hull-Sanders *et al.* 2007; Ridenour *et al.* 2008; Caño *et al.* 2009; Huang *et al.* 2010; Oduor *et al.* 2011).

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While many previous studies investigated invader trade-offs between defence and growth and some addressed changes in secondary metabolites, as mentioned earlier, little information is available for trade-offs among secondary compounds during plant invasions in an evolutionary context.

Plant chemical defences vary in their effectiveness against different herbivores (van der Meijden 1996; Lankau 2007). For example, lignins and tannins are considered especially important in defence against specialists as digestibility-reducing compounds (Müller-Schärer Schaffner & Steinger 2004). Toxins such as alkaloids or flavonoids are considered primarily effective against generalists because specialists may be able to detoxify or sequester such compounds (Müller-Schärer Schaffner & Steinger 2004; Cipollini *et al.* 2008; Napal *et al.* 2010). In addition, chemicals such as flavonoids may have a range of activities beyond herbivore defence, such as defence against microbes or protection against UV damage (Harborne & Williams 2000). Given that invasive plants are not attacked by specialists but may be damaged by novel generalists in their introduced range, it has been predicted (Müller-Schärer, Schaffner & Steinger 2004; Orians & Ward 2010) that the secondary chemical compounds of plants may vary between populations in the introduced and native ranges. Some studies have examined one type of defence and a few have addressed multiple chemical defences simultaneously. However, no study has yet found a trade-off between groups of chemical defences for any invasive plant (see review by Orians & Ward 2010).

In addition, knowledge gaps on invasive plant defence against herbivory also exist for constitutive and inducible chemical defences (Orians & Ward 2010). Constitutive defences are always expressed in a plant, whereas inducible defences are synthesized or mobilized in response to a stimulus, such as herbivore damage or artificial clipping. There is evidence that constitutive and induced defences are often negatively correlated (Koricheva 2002). Since plants may show different inducible defences in response to herbivory by generalist and specialist herbivores (Cipollini, Purrington & Bergelson 2003), the absence of specialists in their introduced range likely affects invasive plant inducibility. Therefore, including different types of chemical defences that may vary in effectiveness against specialists versus generalists and examining their responses to induction by different herbivores in the same study will provide new and broader insights into understanding invasive plant defence mechanisms.

Here, we examine biogeographical variation in chemical defences against herbivory using Chinese tallow (*Triadica sebifera* (L.) Small = *Sapium sebiferum* (L.) Roxb., hereafter '*Triadica*') as a model species. We also examined inducible chemical defence variation among populations from the introduced and native ranges. Previous studies suggest that *Triadica* has evolved to be a faster-growing, less resistant plant in response to low herbivore loads in its introduced range (Siemann & Rogers 2001, 2003b,c; Siemann, Rogers & DeWalt 2006; Zou, Rogers & Siemann 2008a; Huang *et al.* 2010; Wang *et al.* 2011a). Assays for tannin content using the radial diffusion protein precipitation method (Hagerman 1987) with a tannic acid standard showed that populations from the introduced range contain lower concentrations of tannins than native populations, consistent with better performance of specialists on populations from the introduced range than on native ones (Zou *et al.* 2008b; Huang *et al.* 2010; Wang *et al.* 2011a). *Triadica* is known to possess toxins such as flavonoids in its native range (Huo & Gao 2004; Wang, Zhao & Chen 2007; Peng, Yi & Cheng 2008); however, differences in flavonoids between native and introduced *Triadica* populations remain unknown. Flavonoids have other functions including UV resistance, drought resistance and fungal resistance (Harborne & Williams 2000; Cipollini *et al.* 2008; Napal *et al.* 2010). Furthermore, no information is available regarding the inducibility of any types of the defences in either introduced or native populations of *Triadica*.

We investigated the following questions: (i) Is there a trade-off between tannins (especially important for defence against specialists) and flavonoids (especially important for defence against generalists)? We predicted higher flavonoids and lower tannins in populations from the introduced range where specialist herbivores are absent. (ii) How do changes in chemical defences affect specialist and generalist performance on plants from the introduced and native ranges? We predicted that lower tannins and higher flavonoids in plants from the introduced range would have a positive effect on specialists, but not generalists and (iii) Do plants from the introduced and native ranges differ in their inducibility and does induction depend on type of herbivore?

Materials and methods

STUDY ORGANISMS

Native to China and Japan, *Triadica* is a very common perennial tree (Zhang & Lin 1994). It was first introduced to Georgia, USA, in the late 18th century for agricultural and ornamental purposes (Bruce *et al.* 1997). Currently it is listed as a noxious invasive weed in Florida, Louisiana, Mississippi and Texas (USDA/NRCS 2012). *Triadica* is predicted to have the potential of spreading 500 km northwards beyond current invaded areas (Pattison & Mack 2008) and is predicted to increase in abundance in many southern forests (Wang *et al.* 2011b). It aggressively displaces native plants and forms monospecific stands in the south-eastern USA (Bruce *et al.* 1997; Siemann & Rogers 2003a).

Gadirtha inexacta Walker (Lepidoptera: Noctuidae) is host-specific to *Triadica*, being considered as a potential biological control agent against *Triadica* (Wang, Zhu, Gu, Wheeler, Purcell and Ding, unpublished data). The moth has 4 or 5 generations per year in Hubei province, China. The eggs of the moth overwinter on branches and leaves and hatch in May. Larvae pass through six instars in approximately 15 days, feed on leaves and can cause severe damage, especially during the last three instars.

Cnidocampa flavescens Walker (Lepidoptera: Limacodidae), a generalist defoliator, can also cause serious damage to *Triadica*. The moth has two generations per year in Hubei, overwintering as mature larva in the cocoon. The larva pupates and the adult appears in mid- and late May, respectively. The neonate larvae feed on the lower leaf cuticle, producing small transparent circular patches. Feeding by late instars produces large holes on the leaves. The larvae pass through

seven instars in about 30 days. *C. flavescens* is introduced to the United States but its introduced range does not overlap with that of *Triadica*.

Grammodes geometrica Fabricius (Lepidoptera: Noctuidae) is also a generalist, having three or four generations per year in Hubei. Larvae pass through five instars, feed on leaves and can cause severe damage to *Triadica*, especially after the second instar. The larva is a false looper and could develop to pupa within 15 days.

In this study, larvae of *G. inexacta*, *C. flavescens* and *G. geometrica* were collected in fields in Wuhan from April to June 2010. We reared them on potted *Triadica* (Wuhan population) in the Wuhan Botanical Garden, at the Chinese Academy of Sciences, Hubei, China (30°32′ N, 114°24′ E). The offspring of these collections were used for experiments.

SEEDS AND SEEDLINGS

The experiments were conducted at Wuhan Botanical Garden in 2010. In late November 2009, we collected seeds from eight populations across south China (hereafter referred to as native populations) and eight populations from the south-eastern United States (referred to as introduced populations; Fig. 1). Recent molecular studies indicated that the populations in the introduced range come from at least two distinct introduction events with the original introduction to Georgia and South Carolina likely from a southern China population. Later introductions to the United States are likely from the north-east part of *Triadica*'s range (DeWalt, Siemann & Rogers 2011). Therefore, we consider the populations used in this experiment to be representatively native and introduced.

For each population, seeds were collected from 4 to 10 haphazardly selected *Triadica* trees. To evaluate the potential impacts of seed provisioning on seedling performance, 20 seeds from each population were weighed. Seed weights did not differ significantly between native and introduced populations (nested ANOVA, $F_{1,\ 14} = 1.652$, $P = 0.246$). The seed's waxy coats were removed by soaking in water with laundry detergent (10 g L^{-1}) for 2 days. The seeds were then buried in sand at a depth of 5–10 cm and placed in a refrigerator (4 °C) for 40 days.

On 15 April 2010, seeds of 16 populations were planted and maintained in a glasshouse for 6 weeks. Similar-sized seedlings were selected on 20 June 2010 and transplanted individually into pots (height, 16 cm; diameter, 25 cm) containing growing medium (50% locally collected field soil and 50% sphagnum peat moss) and placed in an outdoor common garden. The seedlings were randomly assigned to different treatments. In the common garden, each plant was enclosed by a nylon cage (100 cm height; 27 cm diameter) to exclude herbivores. We tested seedlings because a previous study suggested that the early seedling stage plays an important role in its invasion success (Bruce *et al.* 1997).

INDUCTION EXPERIMENT

To compare the responses of plants from introduced versus native populations to different types of induction, we used artificial clipping and three different herbivore species to damage the seedlings. The control seedlings received no damage treatment. For induced seedlings, one to three larvae were allowed to damage plants for 2 or 3 days. The number of larvae we used for each seedling depended on their damage rates, which varied because larvae were in different instars at the time of induction treatments and varied in size. *C. flavescens* fed more slowly than the other two herbivores. We removed caterpillars when 25% of leaf area had been consumed, which happened on the second or third day after infesting each seedling. For artificial clipping treatment seedlings, we used scissors to clip 25% of leaf area by removing whole leaves at the base of the stem. We removed leaves in each of the 3 days of induction. We started our induction treatments on August 10 and removed larvae after 2 or 3 days. Eleven days after removing herbivores (24 August), we collected the old (produced before the induction period) and new leaves (produced after the induction period) to conduct insect bioassays and chemical analysis and stored these at 4 °C. To compare induced resistance in different age leaves, we used both old and new leaves for bioassays and chemical analysis. Each treatment was replicated eight times, yielding a total of 640 seedlings (two continents × eight populations × five induction treatments × eight replicates).

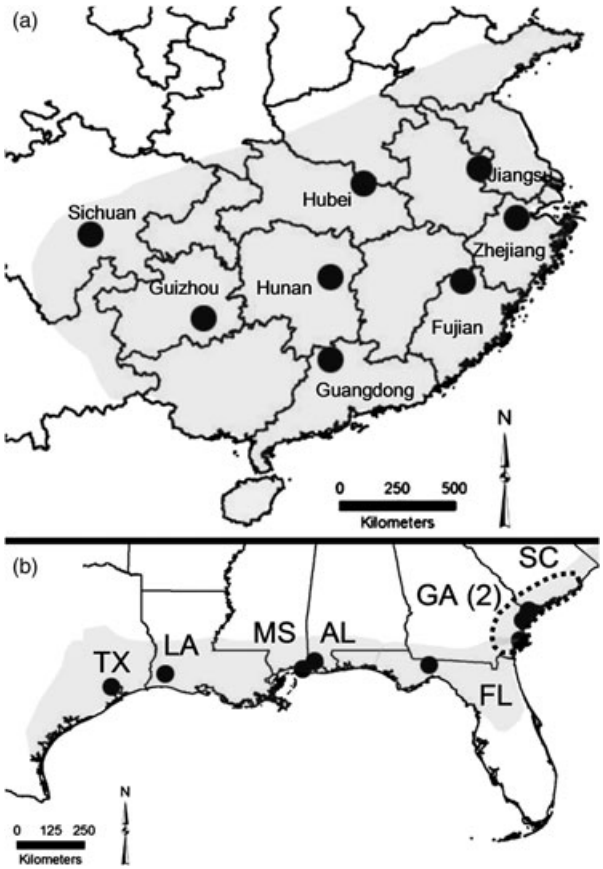

Fig. 1. Native (a) and introduced (b) populations of *Triadica sebifera* that were used in this study. The shaded area in (a) indicates the native range in China and the shaded area in (b) indicates areas in the introduced range with substantial populations (Pattison & Mack 2008). Circles indicate populations where seeds were collected. The dotted area in the introduced range indicates areas that have populations descended from the original introduction, likely from the southern part of the native range in the late 18th century (DeWalt, Siemann & Rogers 2011). The rest of the introduced populations originate from an early 20th century introduction, likely from the Jiangsu province (DeWalt, Siemann & Rogers 2011).

INSECT BIOASSAYS

Leaves of each age and induction treatment combination for each population were randomly assigned to one of the three herbivore

species for feeding trials. In these insect bioassays, we used freshly hatched larvae which had not yet eaten. They were from our maintained colonies, and their weights at egg hatch were assumed to be equal within a species; thus, only final weights were recorded. On the day leaves were collected, one fully expanded leaf was placed on moist filter paper in a Petri dish (inner diameter, 9 cm). A newly hatched larva was transferred to the leaf. Petri dishes were closed and incubated in the laboratory at 24 °C and a 14:10-h light/dark photophase. On the second and third mornings, larvae received a new leaf from the same population and treatment combination. After 72 h, we recorded the mass of each larva. If a larva died within 24 h (only 0.8% of larvae), we repeated the assay with a new larva. Assays were replicated four times (three caterpillar species × two continents× eight *Triadica* populations × two leaf ages × five induction treatments × four replicates = 1920 caterpillars).

CHEMICAL ANALYSES – FLAVONOIDS

Five flavonoids (quercetin, isoquercetin, quercetin glycoside, kaempferitrin and kaempferol) were assessed by high-performance liquid chromatography (HPLC). Quercetin, isoquercetin and kaempferol standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and those for quercetin glycoside and kaempferitrin were obtained from the National Institutes of Food and Drug Control (Beijing, China). All standards had purity ≥97% and were suitable for HPLC. Leaves of each age and induction treatment combination for 10 populations (five China, five US) were dried at 40 °C for 5 days and then ground to a powder. Then, the leaf powder was weighed and soaked for 24 h in a methanol–0.4% phosphoric acid in water solution (48:52, v:v). The solutions were filtered through a 0.22-μm membrane. The filtered extract (20 μL) was injected into a Dionex ultimate 3000 series HPLC (Dionex, Sunnyvale, CA, USA) and compounds were separated on a ZORBAX Eclipse C18 column (4.6 × 250 mm, 5 μm; Agilent, Santa Clara, CA, USA). Flavonoids were eluted at a constant flow of 1.0 mL min^{-1} with a 100% methanol–0.4% phosphoric acid in water gradient as follows: 0–10 min, 48:52; 10–18.5 min, 65:35. UV absorbance spectra were recorded at 254 nm. Concentrations were calculated and standardized by peak areas of standards of known concentrations and then reported as percentage of dry mass for each of the five flavonoids. Total flavonoid concentration, as percentage of dry mass, was calculated as the sum of these five concentrations.

CHEMICAL ANALYSES – TANNINS

Four tannins (gallic acid, catechin, tannic acid and ellagic acid) were assessed by HPLC. All standards were purchased from Sigma-Aldrich, and the purity was ≥95% and suitable for HPLC. Leaves of each age and induction treatment combination for each of 10 populations (five from China, five from United States – the same populations used for flavonoid analyses) were used but seven of the 100 population by treatment combinations had too few leaves for tannin analysis. Leaves were dried at 40 °C for 5 days and then ground to a powder. The leaf powder was weighed and extracted ultrasonically in a 50% aqueous methanol solution for 30 min. The mixture was filtered through a 0.45-μm membrane. The extract was injected (20 μL) into the same HPLC system described above for flavonoid analysis. Tannins were eluted at a constant flow of 1.0 mL min^{-1} with methanol–0.1% phosphoric acid in water gradient as follows: 0–7.5 min, 30:70; 7.5–17 min, 55:45. UV absorbance spectra were recorded at 279 nm for gallic acid, catechin, and tannic acid and at 260 nm for ellagic acid. Concentrations were calculated and standardized by peak areas for standards of known concentrations, then reported as percentage of dry mass for each of the four tannins. Total tannin concentration as percentage of dry mass was calculated as the sum of these four concentrations.

CARBON AND NITROGEN ANALYSIS

To examine the differences of primary compounds among native and introduced populations, we measured total carbon and nitrogen in leaves. Leaves of each age and induction treatment combination for each population were dried at 40 °C for 5 days and then ground. The ground leaves were weighed and analysed for total carbon and nitrogen in an elemental autoanalyzer (Vario MAX CN, Elementar, GmbH, Hanau, Germany).

STATISTICAL ANALYSES

We used mixed model ANOVAS to examine the effects of plant origin, leaf age, and induction treatment and their interactions on chemical concentrations and herbivore masses. We treated population nested in origin as a random effect. We used population (origin) as the error term to test for a significant effect of origin. This corresponds to a test of whether the variation between continents is significantly greater than the variation among populations. We used adjusted means partial difference tests to examine whether treatments differed for predictors with more than two levels. For herbivore bioassays and chemical measurements, leaves were pooled across plants within a population, leaf age and induction treatment. Therefore, we used population averages for all analyses rather than data at the level of individual plants.

We used path analysis (Timothy 1994; Novak 2010) to evaluate how differences in the concentrations of various chemicals between native and introduced populations contributed to the overall effect of plant origin on each caterpillar species. Path analysis uses standardized regression coefficients which indicate how many standard deviations of change in a response variable occur with one standard deviation change in the predictor. The strengths of indirect effects are calculated by multiplying the coefficients of the links within a path of causation. First, we calculated the response of the absolute concentrations of each flavonoid and each tannin along with carbon and nitrogen to changing origin (a dummy variable with China = 0 and US = 1). Then we calculated how the mass of each caterpillar species depended on the concentration of each chemical. For each caterpillar species, we multiplied the coefficient for chemical change by the coefficient to caterpillar response to quantify the contribution of each chemical to the overall caterpillar mass dependence to plant origin. Because we were only interested in relative magnitudes and directions of effects, we did not conduct significance tests of paths and we pooled data among leaf ages and induction treatments. However, large magnitude paths are those that are more significant so the qualitative answers do not depend on such significance tests. All data analyses were performed with the statistical analysis software SAS, ver. 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

TANNINS, FLAVONOIDS AND C/N IN INTRODUCED AND NATIVE POPULATIONS

Plants of introduced populations had lower total tannins and higher total flavonoids than native populations (Fig. 2;

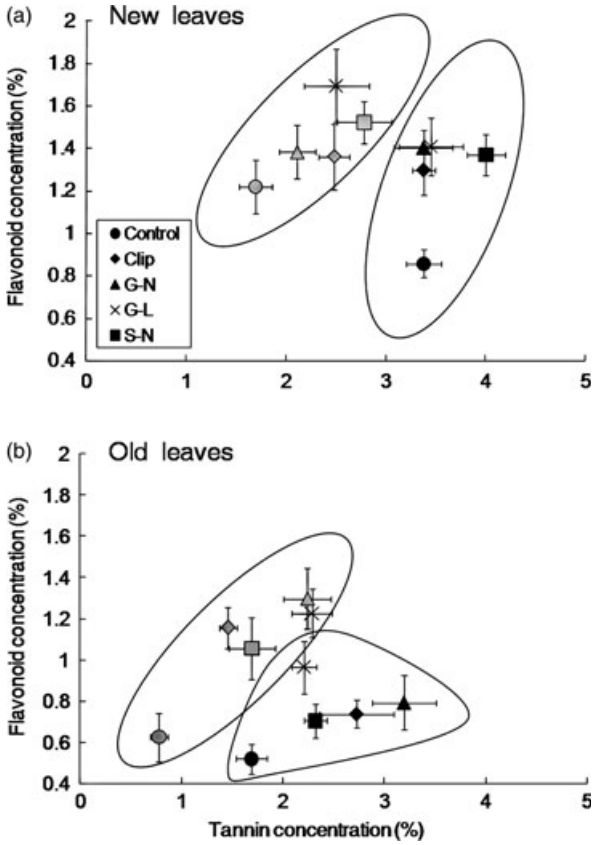

Fig. 2. *Triadica* leaf total tannin and total flavonoid concentrations (% dry wt) in seedlings of native and introduced populations; (a) new leaves and (b) old leaves. Grey symbols represent the introduced populations and black symbols represent the native populations. Different shapes represent different treatments. Each point is the mean of raw data of tannin and flavonoid concentration from all populations of each continent. CLIP, damaged by clipping; G-N, damaged by generalist Noctuidae (*Grammodes geometrica*); G-L, damaged by generalist Limacodidae (*Gadirtha inexacta*): S-N, damaged by specialist Noctuidae (*Gadirtha inexacta*).

Tables 1 and 2 'Continent' factor). This pattern was stronger in new leaves and stronger in induced leaves (Fig. 2; Tables 1 and 2). The form of induction did not have a strong effect on the amounts of total flavonoids or tannins in leaves (Tables 1 and 2). Induction treatment was a significant predictor of flavonoids and tannins but only the control treatment differed from other induction treatments in post hoc tests. No interactive effects of induction and plant origin were significant for total flavonoids or tannins (Tables 1 and 2). However, individual flavonoids or tannins varied in the magnitude of their variation with continent and their responses to induction (Fig. 3a, Tables S1 and S2 in Supporting Information). On average, the concentration of every flavonoid compound was higher and every tannin compound lower in introduced populations than in native populations. Every flavonoid and tannin compound was higher on average in new leaves than in old leaves.

Plant origin (continent) did not affect the carbon concentration (Fig. 3a; Tables 3 and S3 in Supporting Information). Nitrogen concentration was marginally higher in native than in introduced populations ($P = 0.0505$; Tables 3 and S3 in Supporting Information). However, the ratio of C to N (C/N) was significantly higher in introduced populations than in native populations. Both induction treatment and leaf age affected carbon, nitrogen and C/N. There were no significant interactive effects of treatment on C, N or C/N.

SPECIALIST AND GENERALIST CATERPILLAR PERFORMANCE AND THEIR RESPONSE TO INDUCTION

Caterpillar growth varied on leaves that differing in age, plant origin and induction treatments (Fig. 4; Tables 4 and S3 in Supporting Information). Generalist *G. geometrica* biomass was significantly lower when raised on leaves from introduced populations, but specialist *G. inexacta* biomass was significantly higher on invasive plants, and there was no difference for generalist *C. flavescens* biomass. All three species performed better on old leaves (Fig. 4a; Table S3 in Supporting Information). Overall, induction was more strongly associated with a negative effect on the two generalist species and a positive effect on the specialist species (Fig. 4b). Induction by specialist herbivory (S-N) had the most consistent negative effect on caterpillars.

Herbivores varied in their responses to compounds (Fig. 3b). For some compounds, the direction of response of caterpillar growth was consistent across all three species

Table 1. The influence of continental origin of *Triadica* tree populations (Con), induction treatment (Trt), leaf age (Leaf) and their interactions on the concentrations of five flavonoids and total flavonoids in a mixed model ANOVA. Significant values shown in bold

		Kaempferol		Quercetin		Quercetin glycoside		Isoquercetin		Kaempferitrin		Total flavonoids	
Factor	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Con	1,8	1.55	0.2478	9.29	**0.0159**	5.03	0.0552	18.97	**0.0024**	2.61	0.1447	9.89	**0.0137**
Trt	4,72	2.88	**0.0286**	2.91	**0.0272**	9.54	**< 0.0001**	3.67	**0.0089**	4.25	**0.0038**	8.23	**< 0.0001**
Leaf	1,72	20.21	**< 0.0001**	1.37	0.2455	91.16	**< 0.0001**	5.19	**0.0257**	2.81	0.0982	53.75	**< 0.0001**
Con × Trt	4,72	0.01	0.9996	0.32	0.8658	0.02	0.9993	0.45	0.7755	0.26	0.9042	0.01	0.9997
Con × Leaf	1,72	0.03	0.8724	1.00	0.3218	4.38	**0.0400**	0.23	0.6340	1.60	0.2097	1.72	0.1939
Trt × Leaf	4,72	0.55	0.6967	0.49	0.7402	0.52	0.7229	0.99	0.4189	0.45	0.7698	0.39	0.8162
C × T × L	4,72	1.20	0.3192	0.72	0.5824	1.22	0.3098	0.50	0.7324	0.67	0.6121	1.29	0.2823

Table 2. The influence of continental origin of *Triadica* tree populations (Con), induction treatment (Trt), leaf age (Leaf) and their interactions on the concentrations of four tannins and total tannins in a mixed model ANOVA

		Gallic acid		Ellagic acid		Catechin		Tannic acid		Total tannins	
Factor	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Con	1,8	1.31	0.2855	11.21	**0.0101**	7.04	**0.0291**	13.93	**0.0058**	14.07	**0.0056**
Trt	4,60	1.16	0.3366	6.99	**0.0001**	0.39	0.8162	3.51	**0.0122**	3.86	**0.0074**
Leaf	1,60	0.96	0.3306	18.86	**< 0.0001**	0.12	0.7293	32.24	**< 0.0001**	28.95	**< 0.0001**
Con × Trt	4,60	0.70	0.5931	0.99	0.4183	0.66	0.6231	0.79	0.5372	0.80	0.5317
Con × Leaf	1,60	0.07	0.7851	0.00	0.9608	0.02	0.9022	4.87	**0.0312**	3.56	0.0642
Trt × Leaf	4,60	1.22	0.3114	3.37	**0.0150**	0.77	0.5462	1.38	0.2517	1.67	0.1697
C × T × L	4,60	1.10	0.3663	0.82	0.5166	1.66	0.1708	0.22	0.9243	0.38	0.8249

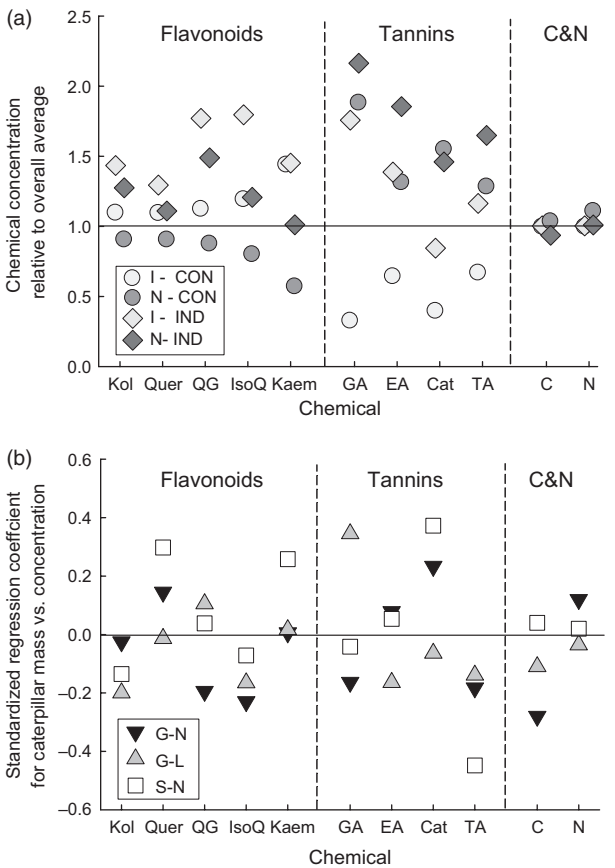

Fig. 3. (a) The influence of continental origin of populations and induction (control versus average of different induction methods) on the concentration of individual compounds. I-CON, not damaged introduced populations; N-CON, not damaged native populations; I-IND, damaged introduced populations; N-IND, damaged native populations. (b) The standardized regression coefficients for the mass of each caterpillar species and the concentrations of different compounds. Kol, kaempferol; Quer, quercetin; QG, quercetin glycoside; IsoQ: isoquercetin; Kaem, kaempferitrin; GA, gallic acid; EA, ellagic acid; Cat, catechin; TA, tannic Acid; C, carbon; N, nitrogen; G-N, generalist Noctuidae (*Grammodes geometrica*); G-L, generalist Limacodidae (*Gadirtha inexacta*): S-N, specialist Noctuidae (*Gadirtha inexacta*).

(negative – Kol, IsoQ, TA; positive – Kaem), but the strength of the effect varied among the three species of caterpillar.

Table 3. The influence of continental origin of *Triadica* tree populations (Con), induction treatment (Trt), leaf age (Leaf) and their interactions on carbon and nitrogen concentrations and their ratio in a mixed model ANOVA

		Carbon		Nitrogen		C:N	
Factor	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Con	1,8	< 0.01	0.9935	5.29	0.0505	6.19	**0.0377**
Trt	4,72	3.72	**0.0083**	11.30	**< 0.0001**	11.13	**< 0.0001**
Leaf	1,72	22.85	**< 0.0001**	12.40	**0.0007**	8.42	**0.0049**
Con × Trt	4,72	0.30	0.8754	1.23	0.3068	1.25	0.2972
Con × Leaf	1,72	0.11	0.7441	1.40	0.2406	2.54	0.1154
Trt × Leaf	4,72	0.69	0.5994	0.91	0.4616	1.09	0.3669
C × T × L	4,72	0.44	0.7784	2.07	0.0939	1.84	0.1305

Table 4. The influence of continental origin of *Triadica* tree populations (Con), induction treatment (Trt), leaf age (Leaf) and their interactions on the mass of the generalist Noctuid (G-N; *Grammodes geometrica*), generalist Limacodid (G-L; *Cnidocampa flavescens*) and specialist Noctuid (S-N; *Gadirtha inexacta*) caterpillars in a mixed model ANOVA

		G-N		G-L		S-N	
Factor	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Con	1,14	10.52	**0.0059**	0.10	0.7573	5.36	**0.0363**
Trt	4,126	10.15	**< 0.0001**	0.93	0.4496	7.52	**< 0.0001**
Leaf	1,126	32.11	**< 0.0001**	4.32	**0.0397**	4.31	**0.0400**
Con × Trt	4,126	3.72	**0.0068**	0.67	0.6132	4.05	**0.0040**
Con × Leaf	1,126	0.94	0.3353	0.28	0.5997	1.99	0.1603
Trt × Leaf	4,126	1.00	0.4096	0.80	0.5276	0.65	0.6303
C × T × L	4,126	3.56	**0.0088**	1.06	0.3809	0.92	0.4550

NET CHANGES IN COMPOUND CONCENTRATIONS AND THEIR EFFECT ON SPECIALISTS AND GENERALISTS

Path analysis showed differences in the concentrations of various compounds between native and introduced populations. There was an increase in concentration for each of the flavonoids from native to invasive range, especially for isoquercetin and quercetin (Table S1 in Supporting Information). In contrast, each of the tannins was lower in the introduced populations, especially for tannic acid and ellagic acid

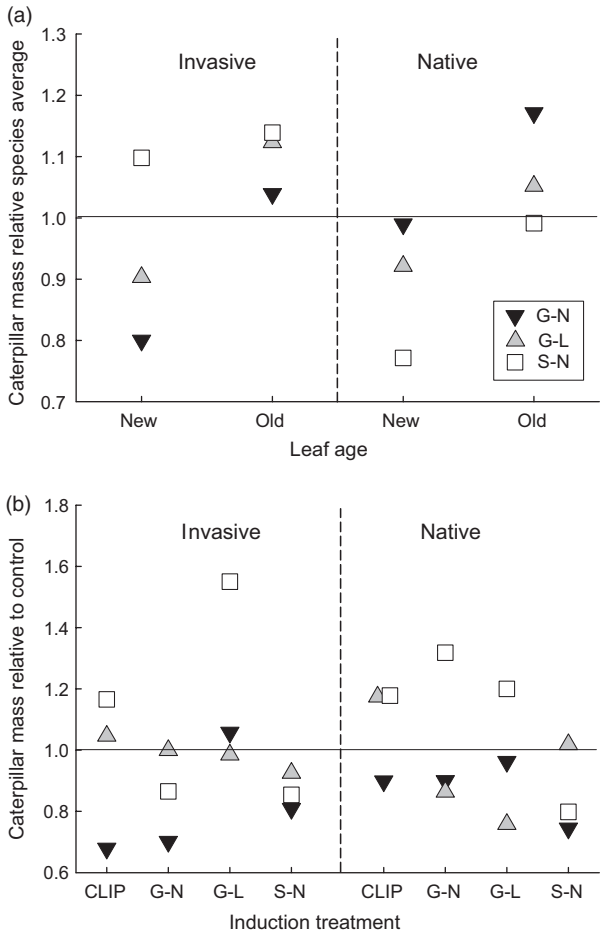

Fig. 4. Larval biomass of three caterpillar species reared on the excised *Triadica* leaves from native and introduced populations; (a) different age leaves and (b) different induction treatments. CLIP, damaged by clipping; G-N, damaged by generalist Noctuidae (*Grammodes geometrica*); G-L, damaged by generalist Limacodidae (*Cnidocampa flavescens*): S-N, damaged by specialist Noctuidae (*Gadirtha inexacta*).

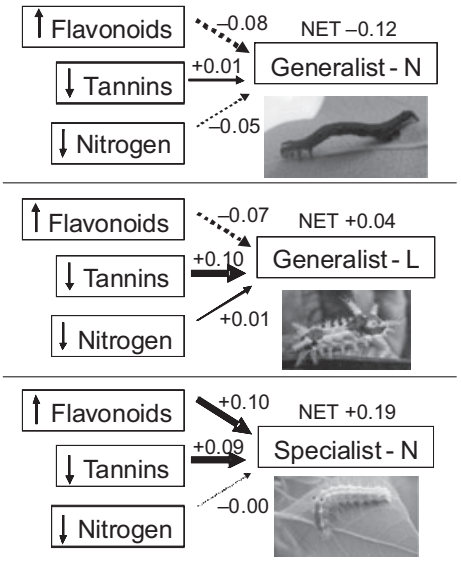

Fig. 5. The results of the path analysis to evaluate how increased flavonoids, decreased tannins and decreased foliar nitrogen in introduced populations compared to native populations contributed to the overall effect of *Triadica* plant origin on each caterpillar species. Solid lines represent positive effects on caterpillar mass from the change and dotted lines negative effects on caterpillar mass. Wider lines represent larger effects. Numbers represent the strength of the effect as a standardized regression coefficient. Generalist-N, Noctuidae (*Grammodes geometrica*); Generalist-L, Limacodidae (*Cnidocampa flavescens*); Specialist-N, Noctuidae (*Gadirtha inexacta*).

(Table S1 in Supporting Information). Both C and N were lower in introduced populations but the change in N was larger (Table S3 in Supporting Information).

The combined net compound changes (flavonoids, tannins and C, N) from native to invasive range negatively affected the generalist *G. geometrica* but positively affected the specialist *G. inexacta* (Fig. 5). The path analysis indicated that the lower performance of the generalist *G. geometrica* was associated with a negative effect of increased flavonoids and lower N but the specialist *G. inexacta* benefited from higher flavonoids and from the lower tannins in introduced populations. Overall, the generalist *C. flavescens* did not have significant changes in mass with origin of *Triadica* leaves, apparently due to offsetting negative effects of increased flavonoids and positive effects of decreased tannins in introduced populations.

Discussion

Invasive plants experience different herbivore interactions in their introduced versus native ranges, so they may vary in defence chemical profiles. In this study, we found *Triadica* plants from the introduced range had lower total tannins but higher total flavonoids compared to plants from native range (Tables 1 and 2; Fig. 2). Individually, we found every flavonoid was higher on average and every tannin was lower on average in introduced populations compared to native populations (Fig. 3; Tables S1 and S2 in Supporting Information), suggesting trade-offs among these secondary compounds. The mass of three caterpillar species that ate these leaves varied from significantly lower (Generalist, *G. geometrica*), unchanged (Generalist, *C. flavescens*), to significantly higher (Specialist, *G. inexacta*) on introduced *Triadica* compared to native *Triadica* (Table 4 and S3 in Supporting Information; Fig. 4). The combined net chemical changes (flavonoids, tannins and C/N) in leaves from native to invasive range plants may underlie these different caterpillar performances (Fig. 5).

CHEMICAL CHANGES IN INVASIVE PLANTS, AND IMPACTS ON SPECIALIST AND GENERALIST HERBIVORES

Tannins are known to have a variety of negative effects on the development and reproduction of herbivorous insects largely due to decreased digistibility of required dietary nutrients (Salminen & Karonen 2011). Specialists and generalists may vary in their responses to tannins based on their level of adaptation to these polyphenolics. In our study, the lower concentration of tannins in the introduced *Triadica* populations was associated with higher larval mass of the specialist, *G. inexacta*

(Table 4; Fig. 5), but generalists varied in their responses to tannins (Table 4; Fig. 5). Lower tannins were associated with higher larval mass of the generalist *C. flavescens* but the generalist *G. geometrica* was apparently not strongly affected by variation in tannins. Our results on tannins and their impact on *G. inexacta* in this study were consistent with our previous reports of lower tannins of introduced *Triadica* populations compared to native populations as measured by the protein precipitation assay (Hagerman 1987; Siemann & Rogers 2001; Huang *et al.* 2010).

We found no significant differences in C or N concentration between introduced and native populations, but C/N was higher in invasives than natives (Table 3; Fig. 3). These results were consistent with our earlier studies of *Triadica* foliar nutrients (Siemann & Rogers 2001; Huang *et al.* 2010). Despite the lack of significant variation in nitrogen related to origin ($P = 0.0505$), the lower mass of the generalist *G. geometrica* when fed leaves from introduced populations appeared to be influenced by nitrogen concentration. The mass of *G. geometrica* also appeared sensitive to the carbon content of leaves but carbon did not vary with origin ($P = 0.99$). Higher foliar nitrogen is generally associated with rapid plant growth and low nitrogen use efficiency (Molinari & Knight 2010), so the higher growth rates of introduced populations suggest that there may be another explanation for lower nitrogen in leaves of introduced populations, such as lower concentrations of nitrogen-based defensive compounds.

Flavonoids such as quercetin, isoquercetin, quercetin glycoside and kaempferol are known to have many activities and are toxic to many generalists (Harborne & Williams 2000; Cipollini *et al.* 2008; Napal *et al.* 2010). The higher concentrations of these flavonoids in the introduced *Triadica* populations were associated with reduced larval mass when consumed by the generalists, *G. geometrica* and *C. flavescens,* but higher larval mass of the specialist *G. inexacta*. So the prediction that flavonoids would have strong negative effects on generalists and positive effects on specialists was well supported by our results.

Diet breadth was not a reliable predictor of the mass of caterpillars fed leaves from introduced versus native populations. We only observed larval growth differences for one generalist, *G. grammodes*, but not for the other generalist, *C. flavescens*, suggesting the two generalists vary in response to changes in secondary metabolites. While flavonoids showed similar negative effects on both of the generalists (Fig. 5), *C. flavescens* was more susceptible to tannins than *G. geometrica* (Fig. 5), which may explain its relatively better performance when fed leaves from introduced *Triadica* populations that were lower in tannins. In addition, life-history differences between these caterpillars may account for their different sensitivities because the larval stage of *C. flavescens* is much longer and presumably their consumption rates are much lower than in *G. geometrica*. Nevertheless, this difference indicates that generalist caterpillars may show different responses to secondary compounds. Therefore, one needs to be cautious when making general conclusions about the effect of secondary compounds on generalists if based on a single herbivore species. In fact, previous studies show that *Triadica* is seldom attacked by generalists in the introduced range (Siemann & Rogers 2003a; Lankau, Rogers & Siemann 2004) and that generalist grasshoppers and beetles prefer to feed on leaves of plants from introduced populations compared to those from native populations (Siemann & Rogers 2003b,c). Indeed, these studies with generalists in the introduced range are not consistent with generalist herbivores driving increases in flavonoid concentrations.

Flavonoids are also known to be involved in defence against microbial pathogens, UV resistance, resistance to nematode attack and drought resistance (Harborne & Williams 2000). Each additional function of flavonoids suggests an alternative explanation for selection for increased flavonoids in the introduced range. It is also possible that increased flavonoids in the introduced range simply reflect a lack of specialists that select for lower flavonoids in the native range. Specialists may also limit flavonoids in native populations indirectly because tannins, which are more important in defence against specialists (Figs 3 and 4), share a biochemical synthesis pathway with flavonoids (Barbehenn & Constabel 2011).

Our results show both generalist and specialist caterpillars performed better on old leaves than on new leaves. Relative to the old leaves, the new leaves contained higher concentrations of flavonoids, which might delay the development of generalists, while containing more tannins, which might explain the slow larval growth of the specialists. Thus, new leaves were better defended.

CHANGES IN SECONDARY CHEMICAL DEFENCE IN INVASIVE PLANTS AND EICA HYPOTHESIS

The EICA hypothesis proposed that invasive plants may reallocate resources from defence against natural enemies to growth and reproduction (Blossey & Nötzold 1995) and secondary chemical defences in invasive plants have subsequently received much attention (see review by Bossdorf *et al.* 2005 and recent studies such as Hull-Sanders *et al.* 2007; Ridenour *et al.* 2008; Caño *et al.* 2009; Huang *et al.* 2010; Oduor *et al.* 2011). However, we still have no conceptual framework that accounts for the variation in herbivore responses to different chemical compounds and the multiple functions of these compounds. Such a framework would allow us to predict the changes in secondary chemical defences in invasive plants and the implications for different herbivores. Perhaps this is why the results from tests of the EICA hypothesis to date are so variable. In this study, we found a contrasting pattern for the two classes of secondary metabolites, tannins and flavonoids. Lower tannins in plants from the introduced range suggest the introduced populations of *Triadica* have a lower resistance to herbivores, thus supporting the EICA hypothesis. However, the higher flavonoid concentration in introduced populations of *Triadica* appears to contradict to the hypothesis. A further analysis of costs of those chemical defences and resource allocation in invasive plants may help to better understand their changes during plant invasions and help to increase the mechanistic detail in the EICA hypothesis.

Plant chemical defence involves fitness costs that vary among chemicals depending on biosynthesis costs and the

relationships between their concentrations and herbivore attack (Strauss & Agrawal 1999; Pilson 2000; Strauss *et al.* 2002; Peñuelas *et al.* 2010). Tannins are often considered a costly defence, because their content in plants can be high (up to 5–10% [dry weight] in woody plants) (Müller-Schärer, Schaffner & Steinger 2004; Germ *et al.* 2010; Huang *et al.* 2010; Orians & Ward 2010; Peñuelas *et al.* 2010). Our results suggest that *Triadica* is investing more resources in tannin production in the native range. In the introduced range where specialists are absent, the production of tannins in *Triadica* is decreased. Since some tannins share the same biosynthesis pathway with some flavonoids (Barbehenn & Constabel 2011), decreasing tannins may lead to increasing flavonoids, which may be more effective against generalists and which have other beneficial functions. However, for the plants from introduced range, the decreases in tannins we observed were less than the increases in flavonoids (in terms of per cent mass; Fig. 2). This suggests that the invader is shifting some of the resources used for tannin production in the native range to flavonoids and some to growth and reproduction in its introduced range. Our study suggests that the future tests on EICA hypothesis should include multiple chemicals, rather than only one class, because of the potential trade-off between chemicals.

CHEMICAL DEFENCE CHANGES AND THEIR IMPLICATIONS FOR BIOLOGICAL CONTROL

Our findings on the changes in secondary chemistry from native to introduced populations have implications for biological control. Larval growth was greater for specialists fed the plants from introduced range in this study (Fig. 4). Our previous work supported these findings as the specialist *G. inexacta* grew bigger and another specialist, a leaf-rolling weevil, *Heterapoderopsis bicallosicollis* built up larger populations on invasive *Triadica* than on natives (Huang *et al.* 2010; Wang *et al.* 2011a). These differences appear to be due to the decreased tannin content and increased flavonoid content in leaves from invasive plants (Fig. 2). Flavonoid content can also affect specialist insect behaviours since these constituents may attract specialists that either use them as cues to locate and/or to accept the host plant for oviposition and/or feeding (van der Meijden 1996). Therefore, higher flavonoid concentrations in the introduced *Triadica* populations may benefit specialists in their host finding and oviposition in addition to the effects we observed on growth, such that the results of these feeding trials may be conservative in terms of the importance of genetic variation in *Triadica* for biological control success. Therefore, our results provide new insight into why super abundance of some biological control specialists has been found on introduced populations relative to their abundance in the native range (Müller-Schärer, Schaffner & Steinger 2004; Wang *et al.* 2011a).

INDUCTION BY HERBIVORES IN INVASIVE PLANTS

Evidence for chemical induction was found in both introduced and native *Triadica* populations as increased levels of flavonoids and tannins were found in the clipping, generalist and specialist herbivory treatments (Fig. 2, Tables S1–S3 in Supporting Information). In this study, we did not detect a significant difference of induction between plant origins (Tables 1 and 2). Trade-offs between constitutive and inducible defence have been reported in some plants (Koricheva 2002); further work is needed to reveal whether such a trade-off exists in *Triadica*. Since these defences are believed to have a fitness cost, if herbivore damage is lower in introduced range than that in native range, then low constitutive but high inducible defences are expected in invasive plants, allowing plants to save resources for growth and reproduction (Cipollini, Purrington & Bergelson 2003). To date, tests on these predictions are rare; only Cipollini *et al.* (2005) reported a lower constitutive level but greater inducibility of glucosinolates in the introduced populations of *Alliaria petiolata* (Brassicaceae) than those in its native populations.

LIMITATIONS

In this laboratory study, we were not able to test many important aspects of plant herbivore interactions. The role of herbivore choice was not tested in these no-choice feeding trials. The short duration of these trials did not allow direct examination of herbivore fitness or population dynamics, which are critical for understanding their effects on host plant populations. The statistical methods used to infer the responses of herbivores to individual chemicals do not demonstrate causation in terms of effects of chemicals as would direct manipulation of chemical concentrations. However, they do provide evidence that chemical defences of *Triadica* vary between ranges, that herbivore growth is sensitive to such variation and suggest how different defence chemicals contribute to these patterns.

Conclusions

Although many recent studies have examined secondary compounds in invasive plants, most of them only focused on one type of defence and none simultaneously addressed multiple classes of chemical defences. To the best of our knowledge, our study is the first to compare the changes in both types of chemical defences to specialist and generalist herbivores between plants of introduced and native populations. We found opposite patterns of change from native to invasive ranges for tannins and flavonoids, suggesting that there is a trade-off in the production of these two types of defences. Furthermore, our study revealed changes in these chemical defences affect specialist and generalist performance accordingly. However, the mixed results for performance of the two generalists on leaves from introduced versus native populations, together with the analyses that indicated different responses to tannins, suggest that we need to be cautious when making broad conclusions based on only one species. Every type of induction increased both flavonoids and tannins and there were no differences in the inducibility of plants from the introduced versus native range. In conclusion, our results show genetic variation in secondary metabolism in invasive plants and effects of these

changes on herbivores, suggesting divergent selection may favour different types of chemical defences in the introduced range where co-evolved natural enemies, especially specialists, are absent.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The average concentration of flavonoids (% dw) for each combination of continental origin of tallow tree populations, induction treatment and leaf age.

Table S2. The average concentration of tannins (% dw) for each combination of continental origin of tallow tree populations, induction treatment and leaf age.

Table S3. The average concentrations of carbon (ppt), nitrogen (ppt), and caterpillar masses (g) for each combination of continental origin of tallow tree populations, induction treatment and leaf age.

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